TOPICAL TREATMENT WITH PROPOLIS DRESSINGS OF POOR HEALING FOOT ULCERS IN DIABETIC PATIENTS*

P. Temesio [1] (In Memoriam) ; N. Ross [2 §] ; R. Alvarez [3]

[1] Policlínica de Diabetes. Hospital Maciel. Montevideo, Uruguay. [2] APITER Ltda. Carlos Ma. de Pena 4469. 11900 Montevideo, Uruguay. E-mail: apiter@apiter.com [3] Instituto de Estadística, Facultad de Ciencias Económicas. Eduardo Acevedo 1139. Montevideo, Uruguay

Poor healing foot ulcers affect 15% of diabetic patients, precede 84% of all diabetes-related lower leg amputations[(1)] and, compared with diabetic patients without ulcers, the mortality at 3 years is 15% higher[(2)]. Due to impairment in the physiological synchronization of events that lead to a rapid healing, foot ulcers do not follow an orderly and reliable wound healing process [(3, 4)] .

Surprisingly, although propolis has been used to cure wounds since ancient times, documented clinical experiences have scarcely been published. For this reason, we present the results of a small historic observational study performed in 1983 in 23 ambulatory diabetic patients (13 type I, 10 type II) with foot ulcers who received topical treatment with 8% propolis of Uruguayan origin in hydro soluble vehicle dressings. Quantitative analysis of polyphenols in Uruguayan EEP using high performance liquid chromatography – mass spectrometry showed a mean value of 457mg/g, with a high content of flavonoids[(5)]. On day 6 of treatment, two patients were withdrawn from the study and excluded for efficacy analysis due to perilesional allergic reaction, which cleared after 3 days' treatment with topical corticosteroids. A study carried out in 1997 to compare the efficacy and safety of the treatment with 8% versus 2% propolis dressings from Uruguay in patients with burns (n=76), wounds (n=122) or ulcers (n=31) showed no differences as to efficacy, but a significant reduction in local allergic events[(6)].

***Part of this paper was presented at the APIMONDIA Congress held in September 2011 in Buenos Aires, Argentina.**

§ To whom correspondence should be addressed.

8% and 2% propolis dressings were kindly supplied by Laboratorio APITER Ltda.

Abbreviations used: EEP, ethanol extract of propolis; TNF-α, tumor necrosis factor-alpha; IL, interleukin; MCP-1, monocyte chemo-attractant protein-1; PGE_2, prostaglandin E_2; LTB_4 and LTC_4, leukotriene B_4 and C_4; ROS, reactive oxygen species; RNS, reactive nitrogen species; MMPs, matrix metalloproteinases; ECM, extracellular matrix; VEGF, vascular endothelial growth factor; TGF-β_1, transforming growth factor-beta$_1$; NF-kB, nuclear (transcription) factor-kappa B; AMPs, antimicrobial peptides; BK_{Ca}, large conductance calcium-activated potassium channels; CAPE, caffeic acid phenethyl ester; PLA_2, phospholipase-A_2; COX-2, cyclooxigenase-2; LOX-5, lipooxigenase-5; NO, nitric oxide; XO, xanthine oxidase; MPO, myeloperoxidase; NADPH, nicotinamide adenine dinucleotide phosphate.

Characteristics of the 21 patients before treatment were as follows: mean age 48±18.7 years (range 17-77); mean diabetes duration 13.5±7.5 years (range 3-33); mean wound duration 77± 27.7 days (range 30-120). Eleven patients had neuropathic ulcers (2 with underlying lipoidic necrobiosis; 4 with bilateral ulcers); 5 had neuroischemic ulcers, 3 had traumatic wounds and 2 had venous ulcers. For treatment purposes, wounds were fully covered with propolis occlusive dressings (plus a 8% propolis solution in four deep wounds) and replaced every 3 days; surgical debridement was performed as required. Fifteen patients received concomitant antibiotics. Seventeen wounds from 13 patients , documented photographically before and after treatment are shown below (at the top, patients initials, gender, age and diabetes duration are indicated; at the bottom, number of days before and after mean wound duration and time of treatment, respectively). After a mean treatment time of 40.5±16 days (range 18-75) , wounds completely healed in 20 patients and significantly improved in one. Propolis dressings provided for a moist wound environment, facilitated autolytic debridement and healthy granulation tissue formation; were painless and easy to use and to remove without trauma to the wound. In conclusion, propolis dressings were effective, safe and inexpensive to treat poor healing diabetic wounds. It should be noted that in the U.S.A. the costs of treatment of diabetic foot ulcers totaled almost $ 40 billion in 2007[7].

A large amount of work was done to elucidate the molecular and cellular milieu of chronic wounds, with the ultimate goal of removing the main barriers that delay healing [8-10]. These barriers are:1) bacterial burden and a noteworthy biofilm formation; polymicrobial aggregates that developed a successful strategy for increasing virulence, resistance to antibiotics (up to 1000-folds) and phagocytosis, all of which are present in up to 80% of chronic wounds[11] ; 2) ischemia; 3) elevated and prolonged inflammation due to the high numbers of infiltrating neutrophils and macrophages, high levels of proinflammatory cytokines (TNF-α, IL-1β, IL-6) and chemokines (IL-8, MCP-1) and to the increased synthesis of lipid inflammatory mediators (PGE_2, LTB_4, LTC_4); 4) large quantities of toxic ROS and RNS (particularly superoxide anion and peroxynitrite) that increase inflammatory response and tissue damage; 5) high levels of proteases (MMPs, neutrophil elastase) that increase ECM degradation and impair angiogenesis and re – epithelialization resulting from endothelial injury[12] , and inactivation of peptide growth factors (VEGF, TGF-β_1 and others) and/or its receptors[9, 13]. Collectively, these events are capable of suppressing cell migration and proliferation, thereby impairing an adequate process of repair. Apparently, in chronic wounds, the reestablishment of a normal repair pattern by topical propolis is mainly related to the ability of its polyphenols to down regulate the activation of NF-kB - the master key of the genetic regulation of immunity and inflammation[14] - induced by bacterial molecules, inflammatory mediators and ROS/RNS; and with its iron chelating capability[15]. In this context, the efficacy of propolis is supported by its antibacterial/antibiofilm, antiinflammatory, antioxidant and immunomodulating properties.

The amphipatic structure of flavonoids facilitates interactions with bacterial cell membranes and the formation of channels that enable ion leakage, thereby reducing membrane potential and bacterial viability[16, 17]. As virtually all bacterial pathogens require iron to survive and develop virulence factors, reducing its availability by chelating is a valid antipathogenic strategy, particularly against Staphylococcus aureus and Pseudomonas aeruginosa[18, 19], bacteria which are frequently isolated from chronic wounds. Indeed, the ability of propolis as an iron chelator seems to be

the leading cause for significantly reducing biofilm formation by the above-mentioned bacteria[20–22]. In turn, this reduction improves wound healing outcomes, indicating that biofilm is the right target for managing the bioburden barrier of chronic wounds [11] Additionally, the activity of the coagulase and lipase enzymes -both related to host tissue damage- was abrogated by propolis in strains of Staphylococcus aureus and significantly reduced in strains of Staphylococcus spp., respectively[20].

The expression of the highly conserved ancestral human skin AMPs (cathelicidin LL-37, beta Defensins), innate immune system molecules that collectively display broad antimicrobial/antibiofilm and high endotoxin neutralization activities, as well as keratinocytes proliferation and migration, is strongly reduced in the bed of chronic wounds[23-27]. The migration of keratinocytes to the more superficial and differentiated epidermal layers, driven by steadily increasing intracellular calcium concentrations resulting from the opening of BK_{Ca} channels[28], dramatically increases AMPs gene expression in the skin[29]. As several propolis-derived polyphenols and CAPE are pharmacological openers of BK_{Ca} channels[30-32] and display an strong antioxidant activity, they could increase AMPs production and protect BK_{Ca} channels functions challenged by the documented damage caused by sustained hyperglycemia[33] and by the high levels of ROS[34] and RNS[35] present in chronic wounds, thereby preserving AMPs production and facilitating re-epithelialization. Additionally, AMPs participate in the wound repair process[24,36]. Thus, a functional link between propolis and AMPs concerning their antibacterial and wound healing properties cannot be ruled out.

In vitro cellular studies showed that the levels and/or gene expression of skin inflammatory mediators were significantly reduced by propolis and/or some of its components. The synthesis of IL-1β was reduced by 65% with 30 µg/mL of propolis[37], whereas that of PGE_2, LTB_4 and LTC_4 were almost completely inhibited by 20-50 µg/mL[38]. The synthesis of ILs-1β, -6, -8, TNF-α, MCP-1, PGE_2, LTB_4 , LTC_4 and endothelial adhesion molecules was reduced by the flavonoids apigenin[39–43], quercetin[37,42–44], chrysin[37,42,43,45], galangin[43] and kaempferol[37,40,42,43] and by caffeic acid[46] and CAPE[38,47,48]. Also, the enzymes related to the synthesis of lipid mediators derived from arachidonic acid of cells membranes were inhibited by some propolis polyphenols: PLA_2 by quercetin[49], kaempferol[50] and caffeic acid[46]; COX-2 by CAPE[47], apigenin[51], kaempferol[51,52] and quercetin[52]; LOX-5 by CAPE and caffeic acid[48].

NO is a cellular mediator of physiological or pathological events in function of the amount produced and the enzyme involved in its synthesis. High levels of NO produced by the inducible isoform of nitric oxide synthase (iNOS) at micromolar range for hours or days are clearly proinflammatory and detrimental for healing due to the in vivo formation of peroxynitrite and hydroxyl radicals, after reacting with concomitantly produced superoxide anions. iNOS activity and gene expression induced by IL-1β, TNF-α, bacterial molecules and hypoxia are significantly increased in chronic ulcers[53 - 55]. In vitro, 12.5 µg/mL of propolis inhibited NO production by 65% by decreasing iNOS gene expression and directly inhibiting its catalytic activity[56]. Another study showed a 65% reduction of iNOS expression with 30 µg/mL propolis, whereas 30 µM of chrysin, galangin, kaempferol or quercetin displayed stronger reductions [37]. iNOS expression was also dose dependently-down regulated by apigenin, kaempferol and quercetin[42,51,52]**.** Additionally, in concentrations ranging from 5 to 15 µg/mL [57] propolis showed a high ability to scavenge peroxynitrite, a powerful oxidizing and nitrating molecule.

Endothelial cells, senescent fibroblasts, macrophages, and particularly neutrophils are the sources of the overproduction of ROS, which are released within the chronic wound microenvironment, amplifying the unrestrained damage to cell membranes and structural proteins of the ECM. Thus, the disruption of this deleterious cycle is a valid therapeutic strategy to protect the regenerative tissue from damage[58].

ROS are generated mainly by the enzymatic activity of XO, MPO and NADPH oxidase. The activity of XO was almost completely inhibited with 50 µg/mL of propolis, and CAPE accounted for 33% of this inhibition; its scavenging capacity of superoxide anion was close to 100% with 3 µg/mL[59]. In a cellular assay with human endothelial cells XO activity was inhibited by 50% with 2 µg/mL of propolis[60]. In vivo, the increased MPO activity (fourfold) in the wounds of diabetic rats was prevented by the topical application of a single drop with 8 mg of propolis; concomitantly, wound healing rate and re-epithelialization improved[61]. NADPH oxidase is the major source of ROS in endothelial cells activated by cytokines and/or ischemia/hypoxia, but the expression of its Nox 4 isoform in epithelial cells leads to constitutive ROS generation[62]. The antioxidant effect of Uruguayan EEPs with high content of polyphenols was investigated at cellular level using rabbit endothelial cells. EEP with a concentration of 5.3 mg of total polyphenols/mL significantly reduced the NADPH oxidase activity (≈20%) in association with a reduced expression of the Nox 4 isoform (≈30%). In vitro, its ROS scavenging activity measured by the oxygen radical absorption capacity was extremely high (8µmol Trolox equivalents/mg propolis); additionally, this propolis was an effective inhibitor of lipid and protein oxidation [63].

Unchecked proteolytic activity, a cardinal feature of chronic wounds, is responsible, in synergy with ROS, for the increased degradation of ECM proteins, the impaired angiogenesis, and for the suppression of cell proliferation[9,12,13,58]. Among other proteases, high levels of elastase and MMP-9 released from infiltrating neutrophils were documented in the fluids of human chronic wounds[64-66] and in experimental diabetic wounds[67]. Moreover, increased levels of MMP-9 in diabetic foot ulcers were highly predictive of poor healing rate[66], and inhibition of the high levels of MMP-2 and -9 in chronic wounds exudates significantly increased in vitro angiogenesis [68]. Thus, controlling this proteolytic activity is another complementary approach for the treatment of chronic wounds. Propolis showed a strong inhibitory effect on human neutrophil elastase activity (50% inhibition with 2µg/mL) [60] as well as on MMP-9 activity. CAPE was found responsible for the anti-MMP -9 activity (50% inhibition with 1.0-2.0 nmol/mL) [69] and for the reduction of induced MMP-9 expression by inhibiting the function of NF-kB[70]. One single application of propolis to experimental diabetic wounds suppressed the increased level and activity of MMP-9 and reversed the significant decrease in epithelial closure rate[67].

Elevated concentrations of inflammatory mediators, proteases and ROS into the chronic wound cause pain[10], and high levels of PGE_2 and LTB_4 reduce the stimulation threshold of pain receptors, thereby amplifying pain mechanisms[71]. Thus, the local analgesic effect displayed by propolis could be ascribed to the inhibition of the aforementioned events.

The low levels of NO produced by the constitutive endothelial nitric oxide synthase (eNOS) at nanomolar range and released for short periods of time shows the physiological side of NO. eNOS contributes to the proangiogenic program of capillary endothelium by triggering VEGF-induced endothelial cell proliferation and differentiation and, in turn, VEGF increases NO synthesis and activity by increasing eNOS expression [53,55,72]. So, NO from eNOS appears to play a central role in angiogenesis, a pivotal component of wound repair. Indeed, angiogenesis and

wound healing were markedly impaired in mice with eNOS gene disruption[73,74], and eNOS expression was strongly reduced at the wound site in diabetes-impaired skin repair[75]. However, in vivo gene therapy with eNOS promoted wound healing in diabetic mice[76]. On the other hand, several pathophysiological conditions (ischemia, chronic hypoxia) and inflammatory mediators (TNF-α, NO, bacterial lipopolysaccharides) present in human chronic ulcers reduce eNOS expression[77,78]. In such a context, propolis can protect eNOS expression and activity due to its aforementioned abilities but, most importantly, due to the finding that at cellular level Uruguayan propolis, at concentrations ranging from 3.2 to 5.3 mg/mL, were effective for increasing eNOS expression and inhibiting Nox activity that, together, point to an increase in endothelial NO bioavailability [63].

In conclusion, the improvement produced in the broad range of molecular targets involved in healing makes propolis an effective therapeutic tool for the treatment of chronic ulcers.

REFERENCES

1. BREM H and TOMIC-CANIC M. Cellular and molecular basis of wound healing in diabetes. J Clin Invest 117:1219-1222; 2007
2. RAMSEY SD et al. Incidence, outcomes, and costs of foot ulcers in patients with diabetes. Diabetes care 22:382-387; 1999
3. HARTOCH RS et al. Emergency management of chronic wounds. Emerg Med Clin N Am 25:203-221; 2007
4. FALANGA V. Wound healing and its impairment in the diabetic foot. Lancet 366:1736-1743; 2005
5. KUMAZAWA S et al. Antioxidant activity of propolis of various geographic regions. Food Chemistry 84:329-339; 2004
6. FIERRO MORALES and LOPEZ GARBARINO J. Clinical evaluation of a new hypoallergenic formula of propolis in dressings. In: Bee products. Properties, applications and apitherapy Mizrahi and Y Lensky Eds), pp 101-105. Plenum Press, New York, 1996
7. DRIVER VR et al. The costs of diabetic foot: the economic case for the limb salvage team. J Vasc Surg 52 (3Suppl): 17S-22S; 2010
8. ENOCH S and HARDING K. Wound bed preparation: the science behind the removal of barriers. Wounds 15:213-229; 2003
9. MEDINA A et al. Pathophysiology of chronic non healing wounds. J Burn Care Rehabil 26:306-319; 2005
10. CHEN WYJ and ROGERS AA. Recent insights into the causes of chronic leg ulceration in venous diseases and implications in other types of chronic wounds. Wound Rep Reg 15:434-449; 2007
11. WOLCOTT R and DOWD S. The role of biofilms: Are we hitting the right target? Plast Reconstructr Surg 127 (Suppl): 28S-35S; 2011
12. NAKATAMI K et al. Inhibitory effect of serine protease inhibitors on neutrophil-mediated endothelial cell injury. J Leukoc Biol 69:241-247; 2001
13. LAUER G et al. Expression and proteolysis of vascular endothelial growth factor is increased in chronic wounds. J Invest Dermatol 115:12-18; 2000
14. GHOSH S and HAYDEN MS. New regulators of NF-kB in inflammation. Nat Rev Immunol 8:837-848; 2008
15. KOSTYUK V et al. The promise of plant polyphenols as the golden standard skin anti-inflammatory agents. Curr Drug Metab 11:415-424; 2010
16. MIRZOEVA OK et al. Antimicrobial action of propolis and some of its components: the effects on growth, membrane potential and mobility of bacteria. Microbiol Res 152:239-246;1997
17. CUSHNIE TP and LAMB AJ. Antimicrobial activity of flavonoids. Int J Antimicrob Agents 26:343-356; 2005
18. FRIEDMAN DB et al. Staphylococcus aureus redirects central metabolism to increase iron availability. Plos Pathog 2:e87; 2006 DOI 10.1371/journal.ppat.0020087
19. O'MAY CY et al. Iron-binding compounds impair Pseudomonas aeruginosa biofilm formation, especially under anaerobic conditions. J Med Microbiol 58:765-773; 2009
20. SCAZZOCCHIO F et al. Multifactorial aspects of antimicrobial activity of propolis. Microbiol Res 161:327-333; 2006
21. BULMAN Z et al. A novel property of propolis (bee glue): anti-pathogenic activity by inhibition of N-acyl-homoserine lactone mediated signaling in bacteria. J Ethnopharmacol 138:788-797; 2011
22. PATRIQUIN GM et al. Influence of quorum sensing and iron on twitching motility and biofilms formation in Pseudomonas aeruginosa. J Bacteriol 190:662-671; 2008
23. SCHRÓDER JM and HARDER J. Antimicrobial skin peptides and proteins. Cell Mol Life Sci 63:469-486;2006
24. HEILBORN JD et al. The cathelicidin antimicrobial peptide LL-37 is involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium. J Invest Dermatol 120:379-389; 2003

25. OVERHAGE I et al. Human host defense peptide LL-37 prevents bacterial biofilm formation. Infect Immun 76:4176-4182;2008
26. PARK SC et al. The role of antimicrobial peptides in preventing multidrug-resistant bacterial infections and biofilm formation. Int J Mol Sci 12:5971-5992; 2011
27. NIYONSABA F Antimicrobial peptides β-defensins stimulate keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines. J Invest Dermatol 127:594-604; 2007
28. MAURO T et al. Keratinocyte K^+ channels mediate Ca^{2+} -induced differentiation. J Invest Dermatol 108:864-870; 1997
29. HARDER J et al. Differential gene induction of human β-defensins (hBD-1, -2, -3 and -4) in keratinocytes is inhibited by retinoic acid. J Invest Dermatol 123:522-529; 2004
30. NARDI A and OLENSEN SP. BK channels modulators: a comprehensive overview. Curr Med Chem 15:1126-1146; 2008
31. NARDI A et al. Natural modulators of large conductance calcium-activated potassium channels. Planta Med 69:885-892; 2003
32. SHIEH DB et al. Properties of BK_{Ca} channels in oral keratinocytes. J Dental Res 84:468-473; 2005
33. LU T et al. Molecular mechanisms mediating inhibition of human large conductance Ca^{2+} activated K^+ channels by high glucose. Circ Res 99:607-616; 2006
34. TANG XD et al. Reactive oxygen species impair Slo 1 BK channel function by altering the cysteine-mediated calcium sensing. Nat Struct Mol Biol 11:171, 2004
35. LIU Y et al. Peroxynitrite inhibits Ca^{2+}-activated K^+ channel activity in smooth muscle of human coronary arterioles. Circ Res 91:1070-1076; 2002
36. CARRETERO M et al. In vitro and in vivo wound healing-promoting activities of human cathelicidin LL-37. J Invest Dermatol 128:223-236; 2008
37. BLONSKA M et al. Effects of ethanol extracts of propolis (EEP) and its flavones on inducible gene expression in J774A.1 macrophages. J Ethnopharmacol 91:25-30; 2004
38. MIRZOEVA OK and CALDER PC. The effect of propolis and its components on eicosanoid production during the inflammatory response. Protag Leukotr Essent fatty acids 55:441-449; 1996
39. NICHOLAS C et al. Apigenin blocks lipopolysaccharide-induced lethality in vivo and pro-inflammatory cytokines expression by inactivating NF-kB through the suppression of p65 phosphorylation. J Immunol 179:7121-7127;2007
40. KOWALSKI J et al. Effect of apigenin, kaempferol and resveratrol on the expression of interleukin-1β and tumor necrosis factor-α genes in 774.2 macrophages. Pharmacol Rep 57:390-394; 2005
41. GERRITSEN ME et al. Flavonoids inhibit cytokine-induced endothelial cell adhesion protein gene expression. Am J Pathol 147:278-292; 1995
42. COMALADA M et al. Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: analysis of the structure-activity relationship. Biochem Pharmacol 72:1010-1021; 2006
43. LOTITO SB and FREI B Dietary flavonoids attenuate tumor necrosis factor-α – induced adhesion molecule expression in human aortic endothelial cells. J Biol Chem 281:37102-37110; 2006
44. SATO M et al. Quercetin, a bioflavonoid, inhibits the induction of interleukin 8 and monocyte chemoattractant protein-1 by tumor necrosis factor-α in cultured human synovial cells. J Rheumatol 24:1680-1684; 1997
45. WOO KJ et al. Chrysin suppresses lipopolysaccharide-induced cyclooxygenase-2 expression through the inhibition of nuclear factor for IL-6 (NF-IL6) DNA-binding activity. FEBS Lett 579:705-711; 2005
46. SONG HS et al. The effect of caffeic acid on wound healing in skin incised mice. Korean J Physiol Pharmacol 12:343-347; 2008
47. MICHALUART P et al. Inhibitory effects of caffeic acid phenethyl ester on the activity and expression of cyclooxygenase-2 in human oral epithelial cells and in a rat model of inflammation. Cancer Res 59:2347-2352; 1999
48. SUD'INA GF et al. Caffeic acid phenethyl ester as a lipooxygenase inhibitor with antioxidant properties. FEBS Lett 329:21-24;1993
49. LEE TP et al. Effect of quercetin on human polymorphonuclear leukocyte lisosomal enzyme release and phospholipid metabolism. Life Sciences 31:2765-2774; 1982
50. GIL B et al. Accelerated communication: Effects of flavonoids on Naja Naja and human recombinant synovial phospholipases A_2 and inflammatory responses in mice. Life Sciences 54:PL333-PL338; 1994
51. LIAN YC et al. Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. Carcinogenesis 20:1945-1952; 1999
52. GARCIA-MENDIAVILLA V et al. The anti-inflammatory flavones quercetin and kaempferol cause inhibition of inducible nitric oxide synthase, cyclooxygenase-2 and reactive C-protein , and down-regulation of the nuclear factor kappa-B pathway in Chang liver cells. Eur J Pharmacol 557:221-229; 2007
53. SCHWENTKER A and BILLIAR TR. Nitric oxide and wound repair. Surg Clin N Am 83:521-530; 2003
54. PAUTZ A et al. Regulation of the expression of inducible nitric oxide synthase. Nitric Oxide 23:75-93; 2010
55. DONNINI S and ZICHE M . Constitutive and inducible nitric oxide synthase: Role in angio-genesis. Antioxid Redox Signal 4:817-823; 2002
56. SONG YS et al. Ethanol extract of propolis inhibits nitric oxide synthase gene expression and enzyme activity. J Ethnopharmacol 80:155-161; 2002
57. LUO Y et al. Evaluation of the protective effects of Chinese herbs against biomolecule damage induced by peroxynitrite. Biosci. Biotechnol. Biochem 74:1350-1354; 2010

58. EMING SA et al. Inflammation in wound repair: molecular and cellular mechanisms. J Invest Dermatol 127:514-525; 2007
59. RUSSO A et al. Antioxidant activity of propolis: role of caffeic acid phenethyl ester and galangin. Fitoterapia 73 (Suppl. 1):S21-S29; 2002
60. BEYER G and MELZIG F. Effects of propolis on hypoxanthine- xanthine oxidase-induced toxicity in cultivated human cells and on neutrophil elastase activity. Biol Pharm Bull 28:1183-1186; 2005
61. McLENNAN SV et al. The anti-inflammatory agent propolis improves wound healing in a rodent model of experimental diabetes. Wound Rep Reg 16:706-713; 2008
62. MARTYN KD et al. Functional analysis of Nox 4 reveals unique characteristics compared to other NADPH oxidases. Cell Signal 18:69-82; 2006
63. SILVA V et al. Antioxidant activity of Uruguayan propolis : in vitro and cellular assays. J Agric Food Chem 59:6430-6437; 2011
64. YAGER DR et al. Wounds fluids: a window into the wound environment? Int J Low Extrem Wounds 6:262-272; 2007
65. TORISEVA M and KÄHÄRI VM. Proteinases in cutaneous wound healing. Cell Mol Life Sci 66:203-224; 2009
66. LIU Y et al. Increased matrix metalloproteinase -9 predicts poor healing in diabetic foot ulcers. Diabetes Care 32:117-119; 2008
67. McLENNAN SV et al. Propolis, the anti-inflammatory bee hive protectant prevents increased matrix metalloproteinase -9 (MMP -9) levels in wound healing in experimental diabetes. Wound Rep Reg 17:A10-A53, Abstract 173; 2009
68. ULRICH D et al. Effect of chronic wound exudates and MMP -2/-9 inhibition on angiogenesis in vitro. Plast Reconstr Surg 116:539-545;2005
69. JIN UH et al. Caffeic acid phenyl ester in propolis is a strong inhibitor of matrix metalloproteinase -9 and invasion inhibitor: isolation and identification. Clin Chim Acta 362:57-64; 2005
70. CHUNG TW. Novel and therapeutic effect of caffeic acid and caffeic acid phenyl ester on hepatocarcinoma cells: complete regression of hepatoma growth and metastasis by dual mechanism. FASEB J 18:1670-1681; 2004
71. MONCADA S and VANE JR. Pharmacology and endogenous roles of prostaglandin endo peroxides, thromboxane A-2 , and prostacyclin. Pharmacol Rev 30:293-331; 1978
72. FUKUMURA D et al. Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. Proc Nat Ac Sci USA 98:2604-2609; 2001
73. MUROHARA T et al. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. J Clin Invest 101:2567-2578; 1998
74. LEE PC et al. Impaired wound healing and angiogenesis in eNOS-deficient mice. Am J Physiol 277:H1600-H1608; 1999
75. STALLMEYER B et al. Regulation of eNOS in normal and diabetes-impaired skin repair: implications for tissue regeneration. Nitric Oxide 6:168-177;2002
76. LUO JD et al. Gene therapy of endothelial nitric oxide synthase and manganese superoxide dismutase restores delayed wound healing in type I diabetic mice. Circulation 110:2484-2493; 2004
77. BUGA GM et al. Negative feedback regulation of endothelial cell function by nitric oxide. Circ Res 73:808-812; 1993
78. TAI SC et al. Endothelial nitric oxide synthase: a new paradigm for gene regulation in the injured blood vessel. Arterioscler Thromb Vasc Biol 24:405-412; 2004